Relationship Between Ischemia/Reperfusion Injury and the Stimulus of Fibrogenesis in an Experimental Model: Comparison Among Different Preservation Solutions

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ABSTRACT

Background and Aims. Orthotopic liver transplantation (OLT) has been the standard treatment for end-stage acute and chronic liver disease. Ischemia-reperfusion (I/R) injury is one of the major causes of poor graft function early after OLT, and adversely influencing graft and patient survivals. It is unknown whether I/R injury influences liver fibrogenesis.

Materials and Methods. Livers from 25 adult male Wistar rats were randomly assigned into 5 experimental groups according to the preservation solution: saline solution (SS); University of Wisconsin (UW) solution; Fructose 1, 6-biphosphate (FBP); S-Nitroso-N-Acetylcysteine (SNAC): or UW + SNAC (SNAC+UW). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactic dehydrogenase (LDH) were determined in preservation solution samples at 2, 4, and 6 hours. After 6 hours of cold ischemia, ex situ reperfusion was applied to the liver for 15 minutes. Serum AST, ALT, LDH, and renin levels were determined. Fresh liver slices were processed for histological studies, determination of thiobarbituric acid reactive substances, catalase, and glutathione, and expression of TGF-β1 and angiotensin II AT1 receptor.

Results. AST was significantly lower during cold storage with UW than with the older media ($P = .001$); ALT was lower in the FBP group ($P = .023$) and LDH was lower in the FBP and SNAC groups ($P = .007$). After reperfusion, serum AST, ALT, LDH, and TBARS showed no significant differences among the groups. Catalase was significantly lower in the SS and FBP groups ($P = .008$ and $P = .006$, respectively). Compared with UW, glutathione concentrations were significantly higher in SS, FBP, and SNAC 200 ($P = .004$). Renin levels were significantly lower in the FBP group ($P = .022$). No histological signs of preservation injury were observed in the hepatic sample. No expressions were detected of TGF-β1 or AT1 receptor.

Conclusion. In this experimental model of early reperfusion injury, preservation changes related to higher levels of renin, which suggest its role in fibrogenesis. FBP was associated with lower renin levels than other solutions including UW.

ORTHOTOPIC liver transplantation (OLT) has been the standard treatment for patients suffering from acute and chronic liver disease,[1–4] but preservation injury continues to be the major cause of poor graft function or graft loss after the procedure.[5–8] Moreover, it has been suggested that preservation injury relates to hepatitis C virus recurrence.[9]

University of Wisconsin (UW) solution is widely used for organ preservation, but it is expensive and also displays a high viscosity. As ischemia-reperfusion (I/R) injury continues to occur with UW, other solutions have been studied.

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We are currently examining alternatives such as Fructose 1–6 Bisphosphate (FBP) and S-Nitroso-N-Acetylcysteine (SNAC) in experimental models.

I/R injury relates to oxidative stress and release of pro-inflammatory cytokines. We hypothesized that these events can lead to fibrogenesis early after the transplantation. In fact, the fibrogenesis process is regulated by a cascade of cytokines, like angiotensin II and β1 growth factor (TGF-β1).[10,11] This study was designed to determine whether I/R induced fibrogenesis and if other solutions protected against or aggrevated it compared with UW.

MATERIAL AND METHODS

Animals

An experimental study was performed using adult male Wistar rats weighing 300–450 g provided by the Laboratory Animal Reproduction and Experimentation Center (CREAL) at Health Sciences Institute at Federal University of Rio Grande do SuI (UFRGS) and kept at our Animal Experimentation Unit (UEA). This study was performed in accordance with the Guide for Care and Use of Laboratory Animals and approved by our Ethics Committee.

Preservation Solutions

Five solutions were studied: (1) saline solution 0.9% (SS); (2) UW (Viaspan, Bristol-Myers-Squibb, 1000 mL); (3) FBP; (4) synthesized SNAC (Sigma Chemical, St Louis, Mo, United States in an acidic solution of sodium nitrite[12]; and (5) UW + SNAC 200 nmol.

Experimental Design

To have their livers removed, animals were anesthetized with inhalation isofluorane 1.5%. The organs were kept in preservation solutions for 6 hours. The subsequent procedures have been previously published by our group.[13]

Biochemical Variables

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactic dehydrogenase (LDH) were measured in cold preservation solution at 2, 4, and 6 hours as well as intra-cardiac blood. Roche/Hitachi Analyzer and Roche Diagnostics Reagents were used to execute standard methods. Renin (kit Renin Maia, Adautis) was determined in the intra-cardiac blood.

Hepatic Measurement of TBARS, Catalase, and Glutathione

To determine TBARS, the hepatic fragment was warmed with thiobarbituric acid (0.67%) and trichloroacetic acid (10%) for 15 minutes. The ruddy product after refrigeration and centrifugation was quantified using spectrophotometry in 535 nm. Catalase activity was assayed according to the method of Aebi (1983) by measuring the absorbance decrease at 240 nm in 10 μl of supernate using a medium of 20 mmol/L H_2O_2, 0.1% Triton X-100, and 10 mmol/L potassium phosphate buffer, pH 7.0. Enzyme activity was expressed as micromoles of H_2O_2 consumed per minute per milligram protein.[14] Reduced glutathione in the hepatic tissue was evaluated using the DTNB method.[15]

Histological Analysis

A mean of 7 g liver tissue were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. The analyzed parameters included steatosis, hepatocyte vacuolization, neutrophilia, and sinusoidal congestion. All samples were evaluated by 2 blinded pathologists.

Immunohistochemistry

The expressions of TGF β1 and AT1 receptor of angiotensin II (AT1) in liver tissue after reperfusion were determined using immunohistochemistry by 2 observers independently. Where their findings diverged, agreement was reached after discussion. A semiquantitative scale was used to standardize the immunohistochemical evaluation for the subsequent statistical analysis. The expression ratio of TGF β1 and receptor AT1 was scored from 0 to 2: 0, no expression; 1, focal expression; 2, diffuse expression.

Statistics

Statistics were performed using SPSS 15.0 for Windows. Data were presented as mean values ± standard deviation (SD); except as otherwise stated. Quantitative variables first analyzed concerning Gaussian distribution were then subjected to the analysis of variance (ANOVA; parametric) or Kruskal-Wallis test (nonparametric). Categorical variables were analyzed using Fisher exact test. Differences were considered statistically significant when $P < .05$.

RESULTS

During cold preservation, UW showed the highest average release rates of AST, ALT, and LDH. Compared with UW, AST was significantly lower in SS, FBP, and SNAC 200 groups ($P = .001$); ALT was lower in group FBP ($P = .023$) and LDH was lower in FBP and SNAC 200 groups ($P = .007$).

After reperfusion, serum levels of hepatic enzymes (AST, ALT, and LDH) and TBARS were not different among the groups (Table 1). Taking UW as the standard, there were significant differences regarding catalase and glutathione levels using the other solutions. Levels of renin were higher with SNAC + UW.

No preservation injury was observed in any of the 5 groups. Mild and focal hepatic ballooning and focal sinusoidal congestion were noted in all UW, SNAC 200, and SNAC + UW samples. Focal sinusoidal congestion was present in 1 SS and no FBP sample; focal hepatic ballooning was found in 4 SS (n = 4) and in all FBP samples (n = 5). There was steatosis in <30% of hepatocytes in 2 samples of the UW group (n = 2), 1 of the SNAC + UW group (n = 1), and 1 of the FBP group (n = 1). Focal necrosis was reported in 2 preserved samples in the SNAC and 1 in the SS group (n = 1).

Expression of TGF β1 and AT1 receptor was not detected in any sample.

DISCUSSION

I/R injury in liver transplantation is a complex phenomenon that contributes to severe complications like primary nonfunction, primary dysfunction, and nonanastomotic biliary stricture.[16] Preservation solutions seek to minimize injury to the organ. UW is certainly the most used preservation solution for liver transplantation but it is not ideal.[17]

Table 1. Biochemical Variables and Oxidative Stress Markers After Reperfusion in the Experimental Groups

	SS (n = 5)	UW (n = 5)	FBP (n = 5)	SNAC 200 (n = 5)	SNAC + UW (n = 5)	*P*
ALT (IU/L)	774 (±670)	742 (±296)	1569 (±1816)	962 (±611)	1003 (±1161)	.733
AST (IU/L)	681 (±422)	959 (±466)	1397 (±1161)	1153 (±694)	823 (±644)	.587
LDH (IU/L)	6045 (±5997)	6251 (±2276)	9590 (±6799)	7549 (±2921)	7360 (±6119)	.825
TBARS (nmol/L/g liver)	70 (±43)	45.2 (±14.3)	68 (±38)	52.2 (±13.5)	47.6 (±25)	.626
Catalase (U/min/mg)	57 (±21)	80.4 (±14.7)	60 (±7.5)	102.3 (±69.5)	*181.8 (±71.4)	.013
Glutathione (nmol/L/g liver)	623 (65–787)	†5699 (890–17,775)	394 (328–1254)	680 (250–1075.5)	2047 (806.5–2299.5)	.004
Renin (ng/mL/h)	17 (±14)	21 (±8)	12 (±6)	16 (±11)	‡40 (±15)	.014

Note: The statistics average of glutathione values were expressed in median (interquartilic interval), Kruskal- Wallis test. Other variables were mean ± SD (one-way ANOVA) and the Bonferroni correction was used to address the problem of multiple comparisons). $P < .05$* was considered statistically significant.
*It differs significantly from SF and FBP.
†It differs significantly from SF, FBP and SNAC 200.
‡It differs significantly from FBP and SNAC 200.

In the present study, SNAC 200 and FBP solutions were more protective to the organ during 6 hours of cold preservation compared with UW and SNAC + UW solutions. FBP has been reported in some studies to exert protective effects on I/R.[18,19] Our previous studies of SNAC and FBP showed good results.[20,21] When we compared SNAC with FBP for the first time in the present study, we noted SNAC to be as effective as FBP. The superiority of SNAC and FBP compared with UW during cold ischemia in intriguing.

UW solution is known to prevent hypothermic effects such as blunting of cellular edema and intracellular acidosis.[19] Moresco et al reported FBP to be superior to UW in a cold ischemia model. In this study and in that of Mota al[22] were confirmed. Maybe the lower viscosity of FBP in comparison with UW is the main cause for the better results. Thus, it is believed that the lower viscosity in SNAC and FBP may be one of the beneficial features to achieve a more homogeneous organ perfusion and better protection of cellular and endothelial barriers. The protective action of SNAC during cold ischemia still remains obscure, but it may relate to protective effects of nitric oxide after reperfusion.

We have observed a protective role of SS on cold ischemia. A hypothermic process may help organ preservation but it does not explain the results after reperfusion. SS may have some oxidant effect as shown by hypertonic SS working as an immunomodulator of T-cell, neutrophil, and macrophage function.[23]

As expected, no anatomopathological preservation lesions were observed, which may relate to the short reperfusion period. There was no expression of TGF β1 or AT1 receptor in the samples probably because of the short reperfusion period. A longer reperfusion period would be necessary because these cytokines are stimulated later in the cascade of the fibrogenesis process.[24] In contrast, lower renin levels, which are present at outset of the cascade, were observed among livers preserved with FBP and SNAC compared with UW, suggesting that fibrogenesis may be more active with UW than with the other solutions. We observed that the combination of UW + SNAC produced more renin possibly secondary to unfavorable interactions of some of the compounds.

In conclusion, we have suggested that I/R injury induces fibrogenesis, as shown by renin activity levels. To prevent fibrogenesis, FBP and SNAC media were superior to UW solution.

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